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(54) Title: ALLOGENEIC AND XENOGENEIC TRANSPLANTATION

(57) Abstract

A method of promoting, in a recipient mammal, acceptance of a graft from a donor mammal including: inducing tolerance to the graft; implanting the graft into the recipient; and enhancing thymus function in the recipient.

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ALLOGENEIC AND XENOGENEIC TRANSPLANTATION

BACKGROUND OF THE INVENTION

The invention relates to tissue and organ transplantation.

SUMMARY OF THE INVENTION

The invention provides several methods of prolonging or promoting the acceptance or survival of a graft, e.g., by inducing tolerance to foreign antigens on allogeneic or xenogeneic organ grafts. These methods can be used individually or in combination with one another. For example, maintenance or enhancement of thymus function can prolong survival of the graft. The thymus function-maintaining or -enhancing methods of the invention can be combined with one or more other methods of prolonging graft acceptance or survival.

Accordingly, the invention features, a method of promoting, in a recipient mammal, e.g., a primate, e.g., a human, acceptance of an allograft, from a donor mammal, including: inducing tolerance to the allograft in the recipient mammal; (preferably) implanting the donor allograft in the recipient; and maintaining or enhancing thymus function in the recipient.

In preferred embodiments, thymus function is maintained or enhanced: by administering growth hormone, e.g., recombinant human growth hormone, or (if the recipient is a male) administering a substance, e.g., a drug which mimics orchiemectomy.

In some surgical procedures, all or part of the thymus is generally removed. In preferred embodiments, thymus function is maintained by not removing recipient thymic tissue.

In preferred embodiments the thymus function which is enhanced or maintained is that of: the recipient's thymus, a donor thymus, or both the recipient's thymus and a donor thymus.

Tolerance can be induced by a method known to those skilled in the art, or by a method described in one of the above recited U.S. patent applications, e.g., by the administration of a short course of help reducing treatment, as is described in USSN 08/220,371, filed March 29, 1994.

Thus, in preferred embodiments, tolerance is induced by the administration of a short course of help reducing treatment to, e.g., induce tolerance to unmatched antigens on the graft. The recipient can be mismatched at a first locus which affects graft rejection, e.g.. an MHC class I or II locus, or a minor antigen locus, and matched, or tolerant of a mismatch. at a second locus which affects graft rejection, e.g., an MHC class I or II locus, or a minor antigen locus. Matching at the second locus can be achieved by selection of a recipient or donor of the appropriate genotype.

In preferred embodiments, tolerance is induced by a short course of help reducing treatment and: the recipient and donor are matched at a class II locus and the short course of help reducing treatment induces tolerance to unmatched class I and/or minor antigens on the graft. In preferred embodiments, tolerance is induced by a short course of help reducing

treatment and: tolerance to a class II antigen is induced by a method other than a short course of a help reducing treatment, and the short course of help reducing treatment induces tolerance to unmatched class I and minor antigens on the graft.

In preferred embodiments, tolerance is induced by a short course of help reducing treatment and: the duration of the short course of help reducing treatment is approximately equal to or is less than the period required for mature T cells of the recipient to initiate rejection of an antigen after first being stimulated by the antigen (in humans this is usually 8-12 days, preferably about 10 days); in more preferred embodiments, the duration is approximately equal to or is less than two, three, four, five, or ten times the period required for mature T cells of the recipient to initiate rejection of an antigen after first being stimulated by the antigen.

In other preferred embodiments, the short course of help reducing treatment is administered in the absence of a treatment which stimulates the release of a cytokine by mature T cells in the recipient, e.g., in the absence of a steroid drug in a sufficient concentration to counteract the desired effect of the help reducing treatment, e.g., in the absence of Prednisone (17, 21-dihydroxypregna-1, 4-diene-3, 11, 20-trione) at a concentration which stimulates the release of a cytokine by mature T cells in the recipient. In preferred embodiments, the short course of help reducing treatment is administered in the absence of a steroid drug, e.g., in the absence of Prednisone.

In preferred embodiments: the help reducing treatment is begun before or at about the time the graft is introduced; the short course is perioperative, or the short course is postoperative; or the donor and recipient are class I matched.

Tolerance can be also be induced by the implantation of donor hematopoietic stem cells, e.g., by a method described in one or more of the above recited U.S. patent applications, e.g., in USSN 07/838,595, filed February 19, 1992.

Accordingly, in yet other preferred embodiments, tolerance is induced by preferably prior to or simultaneous with introduction of the graft, implanting, e.g., by intravenous injection, into the recipient, donor hematopoietic stem cells, e.g., bone marrow, hematopoietic stem cells, (preferably the hematopoietic stem cells home to a site in the recipient mammal).

In a preferred embodiment, the graft is obtained from a different organ than the hematopoietic stem cells, e.g., a liver or a kidney.

In other preferred embodiments, the method includes: (preferably prior to or at the time of introducing the hematopoietic stem cells into the recipient) depleting, inactivating or inhibiting recipient natural killer (NK) cells, e.g., by introducing into the recipient an antibody capable of binding to NK cells of the recipient, to prevent NK mediated rejection of the swine graft. One source of anti-NK antibody is anti-human thymocyte polyclonal anti-serum.

In other preferred embodiments, the method includes: (preferably prior to or at the time of introducing the hematopoietic stem cells into the recipient) depleting, inactivating or inhibiting host T cell function, e.g., by introducing into the recipient an antibody capable of binding to T cells of the recipient; (preferably prior to or at the time of introducing the thymic tissue into the recipient) depleting, inactivating or inhibiting host $CD4^+$ cell function, e.g., by introducing into the recipient an antibody capable of binding to CD4, or $CD4^+$ cells of the recipient. Repeated doses of anti-NK or anti-T cell antibody may be preferable. Monoclonal preparations can be used in the methods of the invention.

Other preferred embodiments include: the step of creating hematopoietic space, e.g., by one or more of, irradiating the recipient with low dose, e.g., between about 100 and 400 rads, whole body irradiation, administering a myelosuppressive drug to the recipient, or administering anti-class I antibodies to the recipient, to deplete or partially deplete the bone marrow of the recipient; the method includes the a step which creates hematopoietic space and the step is performed prior to introducing the hematopoietic stem cells into the recipient.

Other preferred embodiments include inactivating thymic T cells by one or more of: (preferably prior to hematopoietic stem cell transplantation) irradiating the recipient mammal with, e.g., about 700 rads of thymic irradiation; administering one, or preferably two or more, doses of an anti-T cell antibody; or administering to the recipient a short course of an immunosuppressant as described in USSN 08/220,371, filed March 29, 1994.

Other preferred embodiments include: the step of depleting or otherwise inactivating natural antibodies in the blood of the recipient mammal, e.g., by hemoperfusing an organ, e.g., a liver or a kidney, (obtained preferably from the donor) or administering a drug, e.g., deoxyspergualin (DSG) which inactivates or depletes natural antibodies; the method includes a step which depletes or otherwise inactivates natural antibodies in the blood of the recipient and the step is performed prior to hematopoietic stem cell transplantation.

Tolerance can be induced by the implantation of transduced bone marrow cells to induce tolerance to an antigen, e.g., the methods described in one or more of the above recited U.S. patent applications, e.g., in USSN 08/266,427, filed June 27, 1994.

In another aspect, the invention features a method of promoting, in a recipient mammal, e.g., a primate, e.g., a human, acceptance of a xenograft from a donor mammal, e.g., a swine, e.g., a miniature swine, including: inducing tolerance to the xenograft; (preferably) implanting the donor xenograft in the recipient; and enhancing thymus function in the recipient.

In preferred embodiments, thymus function is maintained or enhanced: by administering growth hormone, e.g., recombinant human growth hormone, or (if the recipient is a male) administering a substance, e.g., a drug which mimics orchiemectomy.

In some surgical procedures, all or part of the thymus is generally removed. In preferred embodiments, thymus function is maintained by not removing recipient thymic

In preferred embodiments the thymus function which is enhanced or maintained is that of: the recipient's thymus, a donor thymus, or both the recipient's thymus and a donor thymus.

Tolerance can be induced by a method known to skilled in the art or by a method described in one of the above-recited U.S. patent applications, e.g., by the implantation of donor hematopoietic stem cells, e.g., the methods described in one or more of the above recited U.S. patent applications, e.g., in USSN 07/838,595, filed February 19, 1992.

Thus, in preferred embodiments tolerance is induced by: preferably prior to or simultaneous with introduction of the graft, implanting, e.g., by intravenous injection, into the recipient, donor hematopoietic stem cells, e.g., bone marrow, cord blood or fetal spleen or liver hematopoietic stem cells (preferably, the hematopoietic stem cells home to a site in the recipient mammal).

In preferred embodiments, the recipient is a primate, e.g., a human, and the swine graft is obtained from a miniature swine.

In a preferred embodiment, the graft is obtained from a different organ than the hematopoietic stem cells, e.g., a liver or a kidney.

In other preferred embodiments, the method includes: (preferably prior to or at the time of introducing the hematopoietic stem cells into the recipient) depleting, inactivating or inhibiting recipient natural killer (NK) cells, e.g., by introducing into the recipient an antibody capable of binding to NK cells of the recipient, to prevent NK mediated rejection of the swine graft. One source of anti-NK antibody is anti-human thymocyte polyclonal anti-serum.

In other preferred embodiments, the method includes: (preferably prior to or at the time of introducing the hematopoietic stem cells into the recipient) depleting, inactivating or inhibiting host T cell function, e.g., by introducing into the recipient an antibody capable of binding to T cells of the recipient; (preferably prior to or at the time of introducing the thymic tissue into the recipient) depleting, inactivating or inhibiting host $CD4^+$ cell function, e.g., by introducing into the recipient an antibody capable of binding to CD4, or $CD4^+$ cells of the recipient. Repeated doses of anti-NK or anti-T cell antibody may be preferable. Monoclonal preparations can be used in the methods of the invention.

In other preferred embodiments, the method includes: (preferably prior to or at the time of introducing the hematopoietic stem cells into the recipient) depleting, inactivating or inhibiting recipient natural killer (NK) cells, e.g., by introducing into the recipient an antibody capable of binding to NK cells of the recipient, to prevent NK mediated rejection of the swine graft. One source of anti-NK antibody is anti-human thymocyte polyclonal anti-serum.

Other preferred embodiments include: the step of creating hematopoietic space, e.g., by one or more of, irradiating the recipient with low dose, e.g., between about 100 and 400

administering anti-class I antibodies to the recipient, to deplete or partially deplete the bone marrow of the recipient; the method includes the a step which creates hematopoietic space and the step is performed prior to introducing the swine hematopoietic stem cells into the recipient.

Other preferred embodiments include inactivating thymic T cells by one or more of: (preferably prior to hematopoietic stem cell transplantation) irradiating the recipient with, e.g., about 700 rads of thymic irradiation; administering one, or preferably two or more, doses of an anti-T cell antibody; or administering to the recipient a short course of an immunosuppressant as described in USSN 08/220,371, filed March 29, 1994.

Other preferred embodiments include: the step of depleting or otherwise inactivating natural antibodies in the blood of the recipient mammal, e.g., by hemoperfusing an organ, e.g., a liver or a kidney, obtained from the donor species, e.g., from a pig, or administering a drug, e.g., deoxyspergualin (DSG) which inactivates or depletes natural antibodies; the method includes a step which depletes or otherwise inactivates natural antibodies in the blood of the recipient and the step is performed prior to hematopoietic stem cell transplantation.

Other preferred embodiments include: the step of introducing into the recipient mammal, donor stromal tissue, e.g., swine stromal tissue, preferably hematopoietic stromal tissue, e.g., fetal liver tissue or thymus tissue. In preferred embodiments: the stromal tissue is introduced simultaneously with, or prior to, the hematopoietic stem cells; the bone marrow cells are introduced simultaneously with, or prior to, any anti-NK or T cell antibody.

Other preferred embodiments include those in which: the donor is a pig and both the graft and the hematopoietic cells; and the antibody is an anti-human thymocyte polyclonal anti-serum, obtained, e.g., from a horse or pig.

Tolerance can be also be induced by the implantation of transduced bone marrow cells to induce tolerance to an antigen, e.g., the methods described in one or more of the above recited U.S. patent applications, e.g., in USSN 08/266,427, filed June 27, 1994.

Accordingly, in yet other preferred embodiments, tolerance is induced by inserting DNA encoding an MHC antigen of the donor species into a hematopoietic stem cell, e.g., a bone marrow hematopoietic stem cell, of the recipient; and allowing the MHC antigen encoding DNA to be expressed in the recipient.

Preferred embodiments include those in which: the cell is removed from the recipient mammal prior to the DNA insertion and returned to the recipient mammal after the DNA insertion; the DNA is obtained from the individual mammal from which the graft is obtained; the DNA is obtained from an individual mammal which is syngeneic with the individual mammal from which the graft is obtained; the DNA is obtained from an individual mammal which is MHC matched, and preferably identical, with the individual mammal from which the graft is obtained; the DNA includes an MHC class I gene; the DNA includes an MHC class II gene; the DNA is inserted into the cell by transduction, e.g., by a retrovirus, e.g., by a

blood cells of the recipient for at least 14, preferably 30, more preferably 60, and most preferably 120 days, after the DNA is introduced into the recipient.

Other preferred embodiments further include the step of introducing into the recipient a graft obtained from the donor, e.g., a liver or a kidney.

In other preferred embodiments, the method includes: (preferably prior to or at the time of introducing the hematopoietic stem cells into the recipient) depleting, inactivating or inhibiting recipient natural killer (NK) cells, e.g., by introducing into the recipient an antibody capable of binding to NK cells of the recipient, to prevent NK mediated rejection of the swine graft. One source of anti-NK antibody is anti-human thymocyte polyclonal anti-serum.

In other preferred embodiments, the method includes: (preferably prior to or at the time of introducing the hematopoietic stem cells into the recipient) depleting, inactivating or inhibiting host T cell function, e.g., by introducing into the recipient an antibody capable of binding to T cells of the recipient; (preferably prior to or at the time of introducing the thymic tissue into the recipient) depleting, inactivating or inhibiting host $CD4^+$ cell function, e.g., by introducing into the recipient an antibody capable of binding to CD4, or $CD4^+$ cells of the recipient. Repeated doses of anti-NK or anti-T cell antibody may be preferable. Monoclonal preparations can be used in the methods of the invention.

Other preferred embodiments include: the step of creating hematopoietic space, e.g., by one or more of, irradiating the recipient with low dose, e.g., between about 100 and 400 rads, whole body irradiation, administering a myelosuppressive drug to the recipient, or administering anti-class I antibodies to the recipient, to deplete or partially deplete the bone marrow of the recipient; the method includes the a step which creates hematopoietic space and the step is performed prior to introducing the genetically engineered hematopoietic stem cells into the recipient.

Other preferred embodiments include inactivating thymic T cells by one or more of: (preferably prior to hematopoietic stem cell transplantation) irradiating the recipient mammal with, e.g., about 700 rads of thymic irradiation; administering one, or preferably two or more, doses of an anti-T cell antibody; or administering to the recipient a short course of an immunosuppressant as described in USSN 08/220,371, filed March 29, 1994.

Other preferred embodiments include: the step of depleting or otherwise inactivating natural antibodies in the blood of the recipient mammal, e.g., by hemoperfusing an organ, e.g., a liver or a kidney, obtained from the donor species, e.g., from a pig, or administering a drug, e.g., deoxyspergualin (DSG) which inactivates or depletes natural antibodies; the method includes a step which depletes or otherwise inactivates natural antibodies in the blood of the recipient and the step is performed prior to hematopoietic stem cell transplantation.

Other preferred embodiments include those in which: the same pig is the donor of both the graft and the hematopoietic cells; and the antibody is an anti-human thymocyte

In another aspect, the invention features a method of promoting, in a recipient mammal, e.g., a primate, e.g., a human, acceptance of an allograft, e.g., a heart allograft, from a donor mammal, including: (preferably) inducing tolerance to the allograft; implanting into the recipient the allograft from the donor; and maintaining thymus function in the recipient.

In some surgical procedures, e.g., heart transplants, all or part of the thymus is generally removed. In preferred embodiments, thymus function is maintained by not removing recipient thymic tissue.

In preferred embodiments the recipient thymus function is enhanced.

In preferred embodiments, thymus function is maintained or enhanced: by administering growth hormone, e.g., recombinant human growth hormone, or (if the recipient is a male) administering a substance, e.g., a drug which mimics orchiemectomy.

Tolerance can be induced by a method known to those skilled in the art, or by a method described in one of the above recited U.S. patent applications, e.g., by the administration of a short course of help reducing treatment, as is described in USSN 08/220,371, filed March 29, 1994.

Thus, in preferred embodiments, tolerance is induced by the administration of a short course of help reducing treatment to, e.g., induce tolerance to unmatched antigens on the graft. The recipient can be mismatched at a first locus which affects graft rejection, e.g., an MHC class I or II locus, or a minor antigen locus, and matched, or tolerant of a mismatch, at a second locus which affects graft rejection, e.g., an MHC class I or II locus, or a minor antigen locus. Matching at the second locus can be achieved by selection of a recipient or donor of the appropriate genotype.

In preferred embodiments, tolerance is induced by a short course of help reducing treatment and: the recipient and donor are matched at a class II locus and the short course of help reducing treatment induces tolerance to unmatched class I and/or minor antigens on the graft. In preferred embodiments, tolerance is induced by a short course of help reducing treatment and: tolerance to a class II antigen is induced by a method other than a short course of a help reducing treatment, and the short course of help reducing treatment induces tolerance to unmatched class I and minor antigens on the graft.

In preferred embodiments, tolerance is induced by a short course of help reducing treatment and: the duration of the short course of help reducing treatment is approximately equal to or is less than the period required for mature T cells of the recipient to initiate rejection of an antigen after first being stimulated by the antigen (in humans this is usually 8-12 days, preferably about 10 days); in more preferred embodiments, the duration is approximately equal to or is less than two, three, four, five, or ten times the period required for mature T cells of the recipient to initiate rejection of an antigen after first being stimulated by the antigen.

In other preferred embodiments, the short course of help reducing treatment is

mature T cells in the recipient, e.g., in the absence of a steroid drug in a sufficient concentration to counteract the desired effect of the help reducing treatment, e.g., in the absence of Prednisone (17, 21-dihydroxypregna-1, 4-diene-3, 11, 20-trione) at a concentration which stimulates the release of a cytokine by mature T cells in the recipient. In preferred embodiments, the short course of help reducing treatment is administered in the absence of a steroid drug, e.g., in the absence of Prednisone.

In preferred embodiments: the help reducing treatment is begun before or at about the time the graft is introduced; the short course is perioperative, or the short course is postoperative; or the donor and recipient are class I matched.

Tolerance can be also be induced by the implantation of donor hematopoietic stem cells, e.g., by a method described in one or more of the above recited U.S. patent applications, e.g., in USSN 07/838,595, filed February 19, 1992.

Accordingly, in yet other preferred embodiments, tolerance is induced by preferably prior to or simultaneous with introduction of the graft, implanting, e.g., by intravenous injection, into the recipient, donor hematopoietic stem cells, e.g., bone marrow, hematopoietic stem cells, (preferably the hematopoietic stem cells home to a site in the recipient mammal).

In a preferred embodiment, the graft is obtained from a different organ than the hematopoietic stem cells, e.g., a liver or a kidney.

In other preferred embodiments, the method includes: (preferably prior to or at the time of introducing the hematopoietic stem cells into the recipient) depleting, inactivating or inhibiting recipient natural killer (NK) cells, e.g., by introducing into the recipient an antibody capable of binding to NK cells of the recipient, to prevent NK mediated rejection of the swine graft. One source of anti-NK antibody is anti-human thymocyte polyclonal anti-serum.

In other preferred embodiments, the method includes: (preferably prior to or at the time of introducing the hematopoietic stem cells into the recipient) depleting, inactivating or inhibiting host T cell function, e.g., by introducing into the recipient an antibody capable of binding to T cells of the recipient; (preferably prior to or at the time of introducing the thymic tissue into the recipient) depleting, inactivating or inhibiting host $CD4^+$ cell function, e.g., by introducing into the recipient an antibody capable of binding to CD4, or $CD4^+$ cells of the recipient. Repeated doses of anti-NK or anti-T cell antibody may be preferable. Monoclonal preparations can be used in the methods of the invention.

Other preferred embodiments include: the step of creating hematopoietic space, e.g., by one or more of, irradiating the recipient with low dose, e.g., between about 100 and 400 rads, whole body irradiation, administering a myelosuppressive drug to the recipient, or administering anti-class I antibodies to the recipient, to deplete or partially deplete the bone marrow of the recipient; the method includes the a step which creates hematopoietic space

Other preferred embodiments include inactivating thymic T cells by one or more of: (preferably prior to hematopoietic stem cell transplantation) irradiating the recipient mammal with, e.g., about 700 rads of thymic irradiation; administering one, or preferably two or more, doses of an anti-T cell antibody; or administering to the recipient a short course of an immunosuppressant as described in USSN 08/220,371, filed March 29, 1994.

Other preferred embodiments include: the step of depleting or otherwise inactivating natural antibodies in the blood of the recipient mammal, e.g., by hemoperfusing an organ, e.g., a liver or a kidney, (obtained preferably from the donor) or administering a drug, e.g., deoxyspergualin (DSG) which inactivates or depletes natural antibodies; the method includes a step which depletes or otherwise inactivates natural antibodies in the blood of the recipient and the step is performed prior to hematopoietic stem cell transplantation.

Tolerance can be induced by the implantation of transduced bone marrow cells to induce tolerance to an antigen, e.g., the methods described in one or more of the above recited U.S. patent applications, e.g., in USSN 08/266,427, filed June 27, 1994.

"Enhancing thymus function", as used herein, refers to increasing the ability of recipient or donor thymus to mediate the induction or maintenance of tolerance.

"Maintaining thymus function", as used herein, refers to procedures which maintain the ability of recipient or donor thymus to mediate the induction or maintenance of tolerance.

"Tolerance", as used herein, refers to the inhibition of a graft recipient's immune response which would otherwise occur, e.g., in response to the introduction of a nonself MHC antigen into the recipient. Tolerance can involve humoral, cellular, or both humoral and cellular responses.

"Hematopoietic stem cell", as used herein, refers to a cell, e.g., a bone marrow cell which is capable of developing into a mature myeloid and/or lymphoid cell. Stem cells derived from the cord blood of the recipient or the donor can be used in methods of the invention. See U.S. Patent 5,192,553, hereby incorporated by reference, and U.S. Patent 5,004,681, hereby incorporated by reference.

An immunosuppressive agent capable of inactivating thymic or lymph node T cells", as used herein, is an agent, e.g., a chemical agent, e.g., a drug, which, when administered at an appropriate dosage, results in the inactivation of thymic or lymph node T cells. Examples of such agents are cyclosporine, FK-506, and rapamycin. Anti-T cell antibodies, because they are comparatively less effective at inactivating thymic or lymph node T cells, are not preferred for use as agents. An agent should be administered in sufficient dose to result in significant inactivation of thymic or lymph node T cells which are not inactivated by administration of an anti-T cell antibody, e.g., an anti-ATG preparation. Putative agents, and useful concentrations thereof, can be prescreened by *in vitro* or *in vivo* tests, e.g., by administering the putative agent to a test animal, removing a sample of thymus or lymph node tissue, and testing for the presence of active T cells in an *in vitro* or *in vivo* assay. Such

"Short course of a immunosuppressive agent", as used herein, means a transitory non-chronic course of treatment. The treatment should begin before or at about the time the treatment to induce tolerance is begun, e.g., at about the time, xenogeneic, allogeneic, genetically engineered syngeneic, or genetically engineered autologous stem cells are introduced into the recipient. e.g., the short course can begin on the day the treatment to induce tolerance is begun, e.g., on the day, xenogeneic, allogeneic, genetically engineered syngeneic, or genetically engineered autologous stem cells are introduced into the recipient or the short course can begin within 1, 2, 4, 6, 8, or 10 days before or after the treatment to induce tolerance is begun, e.g., within 1, 2, 4, 6, 8, or 10 days before or after xenogeneic, allogeneic, genetically engineered syngeneic, or genetically engineered autologous stem cells are introduced into the recipient. The short course can last for: a period equal to or less than about 8-12 days, preferably about 10 days, or a time which is approximately equal to or is less than two, three, four, five, or ten times the 8-12 or 10 day period. Optimally, the short course lasts about 30 days. The dosage should be sufficient to maintain a blood level sufficient to inactivate thymic or lymph node T cells. A dosage of approximately 15 mg/kg/day has been found to be effective in primates.

"Lymph node or thymic T cell", as used herein, refers to T cells which are resistant to inactivation by traditional methods of T cell inactivation, e.g., inactivation by a single intravenous administration of anti-T cell antibodies, e.g., anti-bodies, e.g., ATG preparation.

"Help reduction", as used herein, means the reduction of T cell help by the inhibition of the release of at least one cytokine, e.g. , any of IL-2, IL-4, IL-6, gamma interferon, or TNF, from T cells of the recipient at the time of the first exposure to an antigen to which tolerance is desired. The inhibition induced in a recipient's T cell secretion of a cytokine must be sufficient such that the recipient is tolerized to an antigen which is administered during the reduction of help. Although not being bound by theory, it is believed that the level of reduction is one which substantially eliminates the initial burst of IL-2 which accompanies the first recognition of a foreign antigen but which does not eliminate all mature T cells, which cells may be important in educating and producing tolerance.

"A help reducing agent", as used herein, is an agent, e.g., an immunosuppressive drug, which results in the reduction of cytokine release. Examples of help reducing agents are cyclosporine, FK-506, and rapamycin. Anti-T cell antibodies, because they can eliminate T cells, are not preferred for use as help reducing agents. A help reducing agent must be administered in sufficient dose to give the level of inhibition of cytokine release which will result in tolerance. The help reducing agent should be administered in the absence of treatments which promote cytokine, e.g., IL-2, release. Putative agents help reducing agents can be prescreened by *in vitro* or *in vivo* tests, e.g., by contacting the putative agent with T cells and determining the ability of the treated T cells to release a cytokine, e.g., IL-2. The inhibition of cytokine release is indicative of the putative agent's efficacy as a help reducing

assay. In a kidney transplant assay a putative help reducing agent is tested for efficacy by administering the putative agent to a recipient monkey and then implanting a kidney from a class II matched class I and minor antigen mismatched donor monkey into the recipient. Tolerance to the donor kidney (as indicated by prolonged acceptance of the graft) is indicative that the putative agent is, at the dosage tested, a help reducing agent.

"Short course of a help reducing agent", as used herein, means a transitory non-chronic course of treatment. The treatment should begin before or at about the time of transplantation of the graft. Alternatively, the treatment can begin before or at about the time of the recipient's first exposure to donor antigens. Optimally, the treatment lasts for a time which is approximately equal to or less than the period required for mature T cells of the recipient species to initiate rejection of an antigen after first being stimulated by the antigen. The duration of the treatment can be extended to a time approximately equal to or less than two, three, four, five, or ten times, the period required for a mature T cell of the recipient species to initiate rejection of an antigen after first being stimulated by the antigen. The duration will usually be at least equal to the time required for mature T cells of the recipient species to initiate rejection of an antigen after first being stimulated by the antigen. In pigs and monkeys, about 12 days of treatment is sufficient. Experiments with cyclosporine A (10 mg/kg) in pigs show that 6 days is not sufficient. Other experiments in monkeys show that IL-2 administered on day 8, 9, or 10 of cyclosporine A treatment will result in rejection of the transplanted tissue. Thus, 8, 9, or 10 days is probably not sufficient in pigs. In monkeys, a dose of 10 mg/kg cyclosporine with a blood level of about 500-1,000 ng/ml is sufficient to induce tolerance to class II matched class I and minor antigen mismatched kidneys. The same blood level, 500-1,000 ng/ml, is sufficient to induce tolerance in pigs. Long-term administration of 5mg/kg prevents rejection (by long term immune suppression) but does not result in tolerance. 0

"MHC antigen", as used herein, refers to a protein product of one or more MHC genes; the term includes fragments or analogs of products of MHC genes which can evoke an immune response in a recipient organism. Examples of MHC antigens include the products (and fragments or analogs thereof) of the human MHC genes, i.e., the HLA genes. MHC antigens in swine, e.g., miniature swine, include the products (and fragments and analogs thereof) of the SLA genes, e.g., the DRB gene.

"Miniature swine", as used herein, refers to wholly or partially inbred animal.

"Graft", as used herein, refers to a body part, organ, tissue, or cells. Grafts may consist of organs such as liver, kidney, heart or lung; body parts such as bone or skeletal matrix; tissue such as skin, intestines, endocrine glands; or progenitor stem cells of various types.

"A discordant species combination", as used herein, refers to two species in which hyperacute rejection occurs when a graft is grafted from one to the other. Generally,

order. For example, rats and mice are non-discordant species, i.e. their MHC antigens are substantially similar, and they are members of the same order, rodentia.

"Stromal tissue", as used herein, refers to the supporting tissue or matrix of an organ, as distinguished from its functional elements or parenchyma.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DETAILED DESCRIPTION

The drawings will first be briefly described.

Drawings

Figures 1A-1C show a representative clinical course of SLA^{dd} swine which receive SLA^{gg} kidney transplants. Figure 1A shows creatinine levels from animal #10348 (o) which was not treated with cyclosporin A (CyA). This animal rejected the kidney on POD 8, while non-thymectomized CyA-treated animal #10349 (■) went on to tolerate the kidney long-term. The two partially thymectomized pigs #10233 (•) and #10310 (o), as shown in Figure 1B, developed a strong rejection crisis after the end of the CyA treatment, but recovered a normal creatinine on POD 35 and 50, respectively. In comparison, the sham thymectomized animal #10418 (■) maintained a normal renal function during the entire followup. The two completely thymectomized pigs #10549 (o) and #10770 (•), as shown in Figure 1C, developed an irreversible rejection crisis after cessation of CyA.

Figures 2A-2D show the results from cell-mediated lymphocytotoxicity assays performed as follows: SLA^{dd} PBL from each experimental animal were stimulated by outbred cells (Yucatan) and tested on SLA Yucatan target cells (•) as positive control and on SLA^{dd} target cells as negative control (■). SLA^{dd} PBL of the same animals were stimulated by SLA^{gg} matched cells and tested on SLA^{gg} target cells (o). Figure 2A shows representative CML for a non-thymectomized CyA-treated animal #10349. Figure 2B shows representative CML for a sham-thymectomized animal (#10418). Figure 2C shows representative CML for a partially thymectomized animal #10310 and Figure 2D shows representative CML for a completely thymectomized animal #10549.

Figures 3A-3B show an assessment of cytokine expression in serial kidney biopsies from sham, partially and totally thymectomized animals. Figure 3A shows a Northern blot of mRNA derived from kidney biopsy tissue from partially (#10233) and totally (#10770) thymectomized animals on the PODs indicated and developed with probes for IFNγ and the housekeeping gene GAPDH. Figure 3B shows densitometer readings for each of the IFNγ bands, corrected for the amount of RNA loaded onto the gel as determined by the intensity of GAPDH labeling. (*) this RNA sample was degraded.

Figures 4A-4C show densitomer readings, determined as illustrated in Figures 3A and 3B, plotted for labeling of northern blots of mRNA from serial kidney biopsy tissues with probes for IL-10 and IFNγ (compared to the GAPDH bands in each case). (*) no sample.

Figures 4A, 4B, and 4C correspond to data from three separate autoradiographs with various signal intensities, thus leading to different scale for Expression Indices.

The Importance of Thymus Function in Graft Acceptance

Tolerance to renal allografts in miniature swine occurs spontaneously in about one-third of animals selectively matched for class II antigens and mismatched for class I antigens at a single MHC haplotype (Pescovitz, M.D. et al., (1984) *J. Exp. Med.* 160: 1495-1508). Treatment with a short course of Cyclosporine A (CyA, 10-13 mg/kg for the first 12 postoperative days) extended tolerance induction to animals mismatched for class I antigens at both haplotypes and raised the percentage of tolerance induction to 100% (Rosengard, B.R. et al. (1992) *Transplantation* 54:490-497). Acceptors of class I mismatched renal allograft accepted a second kidney transplant from the same or an SLA-matched donor, without further immunosuppression, suggesting a central mechanism of tolerance (Rosengard, B.R. et al. (1994) *Transplantation* 57:794-799). The induction of tolerance may be related to limitation of T cell help at the time of the first exposure of the host's immune cells to class I antigens on the vascular endothelium (Rosengard, B.R. et al. (1992) *Transplantation* 54:490-497). Consistent with this hypothesis, the provision of exogenous T cell help in the form of IL-2 administered during the induction phase of tolerance leads to prompt rejection (Fishbein, J.M. et al. (1993) *Transplant. Proc.* 25:322-323).

The thymus plays an important role in the induction of central tolerance in numerous systems (Kappler, J.W. et al. (1987) *Cell* 49:273-280; Schwartz, R.H. et al. (1989) *Cell* 57:1073-1081; Blackman, M.A. et al. (1990) *Nature* 345:540-542; Sprent, J., H. et al. (1993) *Immunol. Rev.* 133:151-176; Elliot, J.I. (1993) *Immunol. Rev.* 135:215-225; Hugo, P., et al. (1993) *Immunol. Rev.* 135: 133-155; Coutinho, A. et al.(1993) *Immunol. Rev.* 133:225-240; Miller, J.F. (1991) *Transplant. Proc.* 23:9-10). In addition, there are recent reports in rodent models that injections of splenocytes (Nakafusa, Y. et al. (1993) *Surgery* 1 14: 183-190), islets (Posselt, A.M. et al. (1990) *Science* 249: 1293-1295), and bone marrow cells (Odorico, J.S. et al. (1992) *Surgery* 112:370-377) directly into the thymus can induce specific tolerance to allografts. The involvement of the thymus in the induction of tolerance to renal allografts in the swine model, is shown in the experiment described herein in a thymectomy was performed three weeks before kidney transplantation in animals which would otherwise have accepted transplants long-term (i.e. class I mismatched animals treated by the CyA protocol). The data presented herein demonstrate that an intact thymus is required in order for tolerance to be induced by this protocol. In addition, the data indicate that even a small residual amount of thymic tissue is sufficient to permit tolerance to be induced. In contrast, thymectomy in long-term tolerant (LTT) animals does not abrogate kidney allograft tolerance. These experiments are discussed in more detail below.

Effect of thymectomy on the clinical course of CyA treated recipients of class I mismatched kidney transplants

SLA^{dd} swine which receive SLA^{gg} kidney transplants and a 12 day course of CyA (10 mg/kg/day) uniformly accept renal allografts (Rosengard, B.R. et al. (1992). *Transplantation* 54:490-497). These acceptors showed stable renal function after cessation of CyA (creatinine between 1.5-2.0 mg/dl) and no episodes of rejection (Rosengard, B.R. et al. (1992)*Transplantation* 54:490-497). In the present studies these results are reproduced in three additional SLA^{dd} animals (#10349, #10511, #10719), and the clinical course of one representative animal is shown in Figure lA. A sham thymectomized pig (#10418) showed a similar clinical course during the early postoperative period, and proceeded to develop tolerance to the kidney transplant for more than one year, with normal renal function (creatinine < 2mg/dl) (Figure lB). The clinical course for thymectomized pigs was markedly different. Two of these pigs underwent partial thymectomy (#10233, #10310), and developed severe renal dysfunction during postoperative weeks 2-4 (creatinine 15 mg/dl on POD 18 and 11 mg/dl on POD 30, respectively) (Figure 1B). This renal dysfunction was related to a rejection crisis which eventually subsided spontaneously. Histologically however, the infiltration of the kidney allograft by mononuclear lymphocytes was higher to that observed in non-thymectomized CyA-treated animals without renal dysfunction (see below). During this crisis, both animals remained healthy and in good general condition. After this crisis, both pigs became tolerant to the kidney grafts and maintained stable renal function until the day of sacrifice. Pig #10233 had a creatinine of 1.9 mg/dl on POD 330 and pig #10310 a creatinine of 3.2 mg/dl on POD 420. At the time of sacrifice, a complete neck and chest dissection revealed residual thymic tissue in the upper mediastinum in both animals, as demonstrated by microscopic analysis.

Two additional SLA^{dd} pigs (#10549, #10770) underwent a more extensive neck dissection, involving both a cervicotomy and a partial sternotomy. These animals also developed severe renal dysfunction within the first postoperative month, but unlike the two animals which underwent partial thymectomy, the renal function of these animals never returned to normal. Instead, they underwent acute rejection, leading to terminal renal failure. Pig #10549 died on POD 51 with a creatinine of 8 mg/dl and pig #10770 died on POD 26 with a creatinine of 6.3 mg/dl (Figure lC). These two pigs developed massive proteinuria concomitant with a severe hypoproteinemia. Such proteinuria is usually seen in delayed acute rejection and is due to glomerular destruction (Ingulli, E. and A. Tejani (1991) *Transplantation* 51:401-405). Cachexia in these animals probably explains the relatively low creatinine level at the time of death. Although these pigs rejected, the graft survival was prolonged in comparison to the expected survival of less than two weeks for an SLA^{gg} kidney in an SLA^{dd} pig without CyA treatment (Figure lA). At autopsy, despite an extensive dissection of the neck and upper mediastinum, no macroscopic thymic tissue was detected in these two animals, and the histological analysis confirmed the absence of thymus in the excised specimens. The difference in clinical course between animals thymectomized by

these two different procedures appeared therefore to be directly related to the extent of the thymectomy.

Three animals were thymectomized during the maintenance phase of tolerance (#10511, #10719 and #10228) but in this case, the thymectomy did not affect the kidney allograft tolerance. Animal #10719 eventually died 180 days after thymectomy from a respiratory arrest during anesthesia with normal renal function (creatinine 2.6 mg/dl). Animal #10511 died 154 days after thymectomy from abdominal infection with normal renal function (creatinine 1.9mg/dl). Animal #10228 is still alive >140 days after thymectomy with normal renal function (creatinine 1.2 mg/dl).

Transplant donors and recipients were selected from our herd of partially inbred miniature swine at 5-7 months of age. The immunogenetic characteristics of this herd and of intra-MHC recombinant haplotypes have been described previously (Kupiec-Weglinski, J.W. et al. (1983) *Transplant. Proc.* XV:531-534; Sachs, D.H. et al. (1976) *Transplantation* 22:559-567).

Recombinant SLA^{gg} (class I^{c}/II^{d}) animals were used as kidney donors, and SLA^{dd} (class I^{d}/II^{d}) animals as recipients, to achieve a selective two-haplotype class I mismatch. All SLA^{dd} recipient were tested for reactivity to SLA^{gg} in CML prior to surgery and demonstrated specific lysis (25-45%).

Surgery was performed as follows. Kidney transplants were performed between swine bearing a two-haplotype class I plus minor antigen disparity (SLA^{gg} to SLA^{dd}). The details of the surgical procedures have been described elsewhere (Kortz, E.O. et al. (1990) *Transplantation* 49:1142-1149). Placement of an indwelling central venous silastic catheter into an external jugular vein facilitated CyA administration and frequent blood sampling for in vitro assays and for monitoring of renal function (BUN, plasma creatinine) and whole blood CyA levels. Thymectomy was performed 3 weeks prior to the SLA^{gg} kidney transplant in two SLA^{dd} pigs (#10233, #10310) through a low transverse cervicotomy. The pre-tracheal muscles were retracted and the trachea exposed from the cervico-thoracic junction to the mandibula, exposing the thymus, which was then dissected and excised. Due to the fact that residual thymic tissue was found at the time of sacrifice of these two animals, we performed subsequent thymectomies through a combined cervicotomy and partial sternotomy approach. Using this exposure, two SLA^{dd} pigs (#10549, #10770) underwent extensive neck and upper chest dissection. In the neck, the thymus is surrounded by a capsule which simplifies the thymic dissection of the cervical portion. However, in the chest, there is no capsule and the thymus consists of fatty tissue adherent to the pericardium, the pleura and the large vessels (Blin, P.C. and M. Pontois (1972) *Rec. Med. Vet* 4:411-426.). A careful dissection and excision of this tissue was performed using cotton on forceps. The tissue excised from the upper mediastinum represented approximately 20-30% of the total thymus tissue weight. A sham thymectomized pig (#10418) underwent a simple cervicotomy

thymectomy during the maintenance phase of tolerance, LTT SLA^{dd} animals #10719, #10511 and #10228 underwent a complete thymectomy using the combined cervicotomy and sternotomy approach.

Immunosuppression was performed as follows. An intravenous preparation of CyA (Sandimmune, i.v.) was generously provided by Sandoz Pharmaceuticals Corporation, Hanover, NJ. CyA was given each morning as a single daily infusion at a dose of 10-13 mg/kg adjusted to maintain trough levels of 500-800 ng/ml for 12 consecutive days postransplant. The first dose was administered peri-operatively prior to the unclamping of the kidney allograft. Whole blood trough levels were determined by monoclonal radioimmunoassay techniques, and the results were expressed in ng/ml.

Allograft rejection monitoring was performed as follows. The clinical endpoints were survival beyond 100 days or terminal renal failure. Animals that were moribund with profound uremia (Creatinine > 10 mg/dl) were euthanized in accordance with the N.I.H. Guidelines for Animal Care and Use. All animals underwent a complete postmortem examination. A careful examination of the renal vascular anastomoses was made in cases of acute rejection to eliminate possible technical failure. A kidney graft was considered rejected when edema, cyanosis and interstitial hemorrhage were present without any signs of vascular thrombosis due to technical failure, concomitantly with a markedly elevated serum creatinine level (> 10 mg/dl). In case of kidney allograft acceptance, the animals were sacrificed at one year. At autopsy, the chest and neck were carefully inspected, and 6-9 biopsies in the upper mediastinum were performed for pathological detection of residual thymic tissue. The kidney allograft was harvested and also examined histologically.

Histology

Serial kidney biopsies on POD 4,8,11,18, and 30 were performed in all experimental animals. The control animals treated with CyA only or sham thymectomy showed similar histology. An interstitial infiltrate of mononuclear cells was observed as early as POD 4 (5.10% of parenchyma), and this rapidly increased to a 50-60% interstitial infiltrate by POD 8. After POD 30, the infiltrate began to diminish, and decreased slowly to a level of 5-10%, which remained present for more than a year. Focal or patchy tubular epithelial infiltration was also often see during the early postoperative period, but likewise disappeared during long-term follow-up. Vascular changes were absent or minimal, consisting of attachment of few mononuclear cells to the endothelium but without intra-endothelial penetration or vasculitis. The two partially thymectomized animals (#10233 and #10310) showed a similar histological picture to CyA-treated animals during the two first postoperative weeks. During the rejection crisis, the interstitial infiltrate increased (70-90%) but then declined progressively during long-term follow-up. At sacrifice, pig #10233 showed a similar histological pattern to that of non-thymectomized CyA-treated animals, while animal #l0310 showed persistent interstitial infiltrate and evidence of interstitial fibrosis (5-20%) and

crisis. These latter signs were absent in CyA-treated tolerant animals and in the sham-thymectomized animal.

The two completely thymectomized pigs showed clear histological evidence of acute cellular rejection during the first postoperative month, with a significantly higher and persistent interstitial mononuclear infiltrate (70-80%). The most significant difference from the other animals was the presence of severe vasculitis and glomerular hemorrhage in the late specimens (POD 18 and 30). In addition, the tubular infiltration was more marked, with notable tubular injury and interstitial edema. Increased glomerular cellularity was also present, and one animal (#10549) also showed interstitial fibrosis (20%). The three animals which underwent thymectomy during the maintenance phase of tolerance, did not show any histologic evidence of rejection during the entire follow-up. At the time of thymectomy and later, a low (5-20%) interstitial infiltrate of mononuclear cells were observed, but no vascular lesions were noted.

Sequential kidney biopsies on POD 4,8,11,18 and 30 and after 100 days (LIT) were performed and stained with hematoxylin and eosin (H&E). Specimens were examined in a blinded fashion by a renal pathologist who graded rejection on the basis of histologic parameters, including mononuclear cell infiltrate, vasculitis, glomerular sclerosis, and tubulitis.

Skin graft survival in LTT animals

The sham thymectomized control and the unthymectomized CyA treated animal, as well as the two partially thymectomized animals which became LTT, received donor matched SLA^{gg} skin grafts. The grafts were significantly prolonged in all of these animals, as has previously been reported for CyA-treated LTT animals in this model (11.7+/-2.6 vs. 25.6+/-4.8) (Rosengard, B.R. et al. (1992) *Transplantation* 54:490-497). Skin grafts survived 25 and 30 days in the two partially thymectomized animals and 30 days in the sham thymectomized animal. On the other hand, donor class I plus third party (TP) class II mismatched skin grafts were rejected almost as promptly in LTT animals (9.5 +/- 1.8 days) as in naive animals (7.8 +/- 1.0 days). The partially thymectomized animals and the sham thymectomized control rejected TP class II mismatched skin grafts in 8, 10 and 12 days, respectively. Similarly, three weeks after thymectomy, LTT animal #10511 and #10719 were challenged by donor class I plus third party class II (SLA^{cc}) skin grafts. Both animals rejected the skin graft in 9 days and animal #10719 developed a transient but strong rejection crisis after the skin graft rejection (creatinine rose from 1.9 to 6.2 mg/dl), while animal #10511 did not show evidence of renal dysfunction. As in previous reports (Rosengard, B.R. et al. (1991) *Transplantation* 52: 1044-1052), such skin graft rejection produced peripheral sensitization as detected by anti-donor pCTLs, but caused no loss of the renal allograft.

Skin graft techniques were performed as follows. Split-thickness skin grafts (4x4cm) were harvested from donors with a Zimmer dermatome and placed on a graft bed, also

occlusive dressing for the first three postoperative days. The time of rejection was defined as the day on which less than 10% of the skin was viable by criteria of warmth, color and texture.

In vitro cell mediated immunity

Non-thymectomized, CyA-treated recipients of class I mismatched kidneys usually demonstrated specific and indefinite anti-donor unresponsiveness in CML after cessation of CyA (Gianello, P. et al. (1993) *Immunol. Rev.* 133:19-44). Figure 2A shows a representative example of CML activity on POD 30 in one such tolerant animal (#10349). The sham-thymectomized animal (#10418) behaved similarly and showed specific antidonor unresponsiveness during the entire follow-up (Figure 2B). The two partially thymectomized animals developed low anti-donor CML reactivity (8 to 15 % specific lysis) during the rejection crisis, but became specifically unresponsive thereafter (Figure 2C). The two completely thymectomized animals developed strong, specific antidonor reactivity during the rejection crisis, as has previously been seen in untreated rejector pigs, and anti-third party reactivity was maintained (Figure 2D). The three animals which underwent thymectomy during long-term tolerance of kidney allografts, showed specific unresponsiveness to donor antigens prior to skin sensitization.

Isolation of PBL was performed as follows. Freshly drawn, heparinized whole blood was diluted with Hank's buffered saline solution (HBSS) (Gibco/BRL, Grand Island, NY) and the mononuclear cells were obtained by gradient centrifugation using Lymphocyte Separation Medium (LSM) (Organon, Teknika, Durham, NC). The mononuclear cells were washed once with HBSS and contaminating red cells were lysed with ACK buffer (B&B Research Laboratories, Fiskeville, RI). Cells were washed a second time with HBSS and resuspended in tissue culture medium. Cell suspensions were kept at 4°C until used in cellular assays.

Cell-mediated lymphocytotoxicity (CML) assays were performed as follows. Media for CML assays consisted of RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 6% fetal calf serum (FCS), 100U/ml penicillin, 135 μg/ml streptomycin, 50, μg/ml gentamicin, 10 mM HEPES, 2mM 1-glutamine, lmM sodium pyruvate, and $5x10^{-5}$ M β-2 mercaptoethanol. Medium used for the effector phase of CML assays consisted of Basal Medium Eagle (GIBCO) supplemented with 6% serum replacement media (Sigma). CML assays were performed as previously described (Kirkman, R.L. et al. (1979) *Transplantation* 28:24-30). Briefly, lymphocyte cultures containing $4x10^6$ responder and $4x10^6$ irradiated (25 Gy) stimulator peripheral blood lymphocytes (PBL) in 2 ml of medium were incubated for 6 days at 37°C in 7% C02 and 100% humidity. Bulk cultures were harvested, and effector cells were tested on ^{51}Cr labeled blasts. The tests were run at serially diluted ratios (100:1; 50:1; 25:1; 12:1). After 5.5 hours of effector cell incubation with the $5x10^3$ specific targets, the supernatants were harvested and ^{51}Cr release was determined on a gamma

(BRL, Rockville, MD). Baseline levels were measured as the rate of spontaneous release of ^{51}Cr from $5x10^3$ targets. The results were expressed as:

$$\% \text{ specific lysis} = \frac{\text{experimental release (cpm)-spontaneous release (cpm)}}{\text{maximum release(cpm)-spontaneous release(cpm)}} \times 100\ \%$$

Humoral assay

It has previously been reported that approximately 50% of CyA-treated animals develop specific anti-donor cytotoxic IgM but not cytotoxic IgG (Rosengard, B.R. et al. (1992) *Transplantation* 54:490-497). The presence of cytotoxic IgM did not correlate with impaired renal function, and usually disappeared within a few months (Pescovitz, M.D. et al. (1984) *J. E*xp. *Med.* 160: 1495-1508; Rosengard, B.R. et al. (1992) *Transplantation* 54:490-497). The three CyA-treated and the sham-thymectomized animals examined in the present study did not produce cytotoxic IgM or IgG. In contrast, all four thymectomized pigs developed high but transient anti-donor IgM reactivity between POD 1 l and 30 as assessed in vitro by a two step ^{51}Cr release assay (cytotoxic titers of 1/32; 1/32; 1/64 and 1/256 for animals #10233, #10310, #10770 and #10549, respectively). The appearance of these cytotoxic IgM coincided with the rejection crisis in all four cases. No antidonor cytotoxic IgG were identified in these animals, not even during kidney rejection in the two completely thymectomized pigs. No antibodies of any class were detected in animals thymectomized during the long-term maintenance phase of tolerance.

Humoral responses were determined as follows. Animals were tested for the presence of anti-donor or third-party cytotoxic antibody (Ab) by a two-stage complement dependent lymphocytotoxicity assay as previously described (Pescovitz, M.D. et al. (1984) *J. Exp. Med.* 160:1495-1508). The medium used consisted of medium 199 (GIBCO) with 1% FCS added as a protein source. Targets matched to the MHC of the donor kidney were used to test for the presence of Ab. Briefly, 25 µl serial dilutions of sera were performed in 96-well round-bottomed plates. 25 µl of target cells (fresh PBL labeled with ^{51}Cr at a concentration of $2x10^6$/ml) were added to each well and were incubated for 15 minutes at 37°C. Cells were washed once and incubated for 30 minutes at 37°C with rabbit complement (1/8) (Pel-Freez, Brown Deer, WI). Supernatants were collected and counted. Calculations of specific lysis were performed similarly to CML assays (see above). All targets were tested with a positive control (alloantiserum) and a normal pig serum to control for non-specific complement cytotoxicity. To determine whether cytotoxic activity was due to IgG or IgM, sera were incubated either with 0.1M 2-ME (Pescovitz, M.D. et al. (1984) *J. Exp. Med.* 160:1495-1508) or 0.2M DTT (dithiothreitol) (Iwaki, Y. et al. (1988) *Clin. Transplan.* 2:81-84) to eliminate IgM activity prior to assay.

Flow Cytometry was performed as follows. Serial serum samples from experimental animals were analyzed by flow cytometry in order to follow the course of alloantibody production. Polyclonal fluoresceinated goat-anti-swine IgG (γ chain) or IgM (μ chain) antibodies (Kirkegaard & Perry Lab., Gaithersburg, MD) were used as the second reagent after incubation with test sera. Normal pig serum served as a control to ensure specific binding. All steps were performed at 4°C using HBSS containing 0.5% bovine serum albumin (BSA) and 0.5% sodium azide.

<u>Northern Blot Analysis of Cytokine Expression</u>

Patterns of cytokine mRNA expression in serial kidney biopsies from sham, partially and totally thymectomized animals were assessed. Figure 3A shows an example of the bands obtained for IFNγ and GAPDH probes on northern blots of kidney biopsy tissues for one partially thymectomized (#10233) and one totally thymectomized (#10770) animal at several time points post-transplant. A plot of the densitometry readings for these bands, calculated as expression indices for IFNγ relative to GAPDH is shown in Figure 3B. Plots of similarly derived densitometry readings for expression of both IL-10 and IFNγ, each relative to GAPDH, are shown in Figure 4A-4C for two partially thymectomized and two totally thymectomized animals, as well as for a sham thymectomized animal. As seen in this figure, POD 8, both completely thymectomized animals expressed a strong IFNγ signal, and expression of IFNγ remained detectable until rejection of the kidney. Both partially and sham-thymectomized animals showed relatively little or no IFNγ expression at the same time. In contrast, IL-10 expression was relatively high in the partially thymectomized animal #10310 on POD 8,11,18 and 30. Unfortunately, IL-10 message was not measured beyond day 11 in animals #10233 (partially thymectomized) and 10418 (sham thymectomized), but both of these animals showed high IL-10 expression relative to IFNγ expression up to day 11. Animal #10770 (complete thymectomy) expressed a high level of IL-10 on POD 8, but this expression was transient and fell to very low levels over the following week. The sham-thymectomized animal (#10418) expressed a strong signal on POD 8 and 11, with very little expression of IFNγ. Thus, for totally thymectomized animals, the ratio of IFN γ to IL-10 expression was higher at all early time points than it was in partially thymectomized animals or in the sham-thymectomized animal. These results suggest a possible correlation between a Th1 cytokine pattern leading to rejection and a Th2 cytokine pattern leading to the induction of tolerance. This same correlation has been noted for a comparison of cytokine patterns between untreated and CyA-treated non-thymectomized animals.

Northern Blot Analysis was performed as follows. Cytokine gene expression was analyzed by Northern blots on serial kidney biopsies (POD 4,8,11,18 and 30). Renal tissue wedges weighing 150-200 mg were obtained by open biopsies. The excised renal tissue was directly immersed in 3 ml of Guanidine Thiocyanate 4M containing 2% 2-mercapto-ethanol and immediately frozen in liquid nitrogen. RNA extraction was achieved as described

Laboratory Press, N.Y.). The probe for IFN-γ was a cDNA fragment of 0.75 kb obtained from Dr. Lefevre (INRA, Jouy-en-Josas, France), and the IL-10 probe was a cDNA fragment of 0.18 kb generated by PCR from pig DNA using primers chosen from the most homologous portions of human and mouse IL-10 sequences. Northern blot analysis was performed as described (Meinkoth, J. et al. (1984) *Anal. Biochem.* 138:267). Briefly, 20 μg samples of RNA were loaded on 1.2% formaldehyde agarose gels, run overnight at 22 Volts, washed in distilled water twice for 10 min and then incubated in 10X SSC for one hour at room temperature. The gels were then blotted onto Zetaprobe membranes (Bio-Rad) and cross-linked in a UV Stratalinker (Stratagene) by being exposed to 1200 μjoules. Probes were labeled with ^{32}p dATP and dCTP to a specific activity of 10^9 cpm/μg by random priming (Promega). Filters were prehybridized for at least 4 hours in 10% Dextran Sulfate, 4X SSC, 40% Formaldehyde, 5X Denhardt's, 0.5 % SDS, 20 ug/ml salmon sperm DNA, and then hybridized to ^{32}p labeled probes in the same solution at 42°C for 14 hours and developed as autoradiographs. Intensities of radioactive signals were determined with a computing densitometer (Molecular Dynamics, Sunnyvale, CA). Expression indices (EI) were calculated by correcting the levels of signals observed in experimental samples according to the amount of RNA loaded as estimated by the relative intensity of the GAPDH signal. The EI was calculated as follows:

$$EI(I)= \frac{(\text{Value of sample})_{(I)} \times (\text{Highest Value of GAPDH})}{\text{Val. GAPDH}_{(1)}}$$

<u>The Role of the Thymus in Graft Acceptance</u>

It has been previously demonstrated that a 12-day course of CyA permits tolerance to renal allografts to develop in 100% of miniature swine mismatched selectively for two haplotypes at MHC class I, while 100% of similarly matched renal allografts are rejected without CyA treatment (Rosengard, B.R. et al. (1992) *Transplantation* 54:490-497). This striking difference in allograft survival has made in vivo studies feasible to assess the mechanism by which tolerance to primarily vascularized allografts is induced in this large animal model. The treatment of transplanted animals with an exogenous source of IL-2 on post-operative days 8, 9 and 10 in addition to the usual CyA regimen leads uniformly to rejection, supporting the hypothesis that inhibition of cytokines by CyA is probably involved in the mechanism by which tolerance ensues. In the studies described herein, the possible role of the thymus in this mechanism was investigated.

The data indicate that an intact thymus is required in order for tolerance to occur in this system. Partial removal of the thymus led to a severe, although self-limited, acute rejection crisis, while complete thymectomy led to irreversible acute rejection. There are

It is generally accepted that intrathymic recognition of self antigens results in the deletion of high affinity T-cell clones at an early stage of differentiation, and is therefore responsible for self tolerance (Kappler, J.W. et al. (1987) *Cell* 49:273-280). A similar mechanism could be involved in the development of tolerance to alloantigens, if appropriate cells bearing those antigens (possibly dendritic cells) were to relocate from the graft to the thymus following renal transplantation. Clearly, such homing occurs after bone marrow transplantation (Sprent, J. et al. (1993) *Immunol. Rev.* 133:151-176) and leads to tolerance, and the possibility that it also occurs after solid organ transplantation has been suggested by Starzl and colleagues (Starzl, T.E. et al. (1992) *Lancet* 339:1579-1582). There are also several recent reports indicating that alloreactivity can be diminished or eliminated by the direct injection of allogeneic cell populations into the thymus (Nakafusa, Y. et al. (1993) *Surgery* 1(14):183-190; Posselt, A.M. et al. (1990) *Science* 249:1293-1295; Odorico, J.S. et al. (1992) *Surgery* 112:370-377; Goss, J.A. et al. (1993) "Induction of Donor-Specific Transplantation Tolerance with Direct Intrathymic Injection of Splenocyte Alloantigen" *in* The Thymus-Regulator of Cellular Immunity (R.G. Landes Company, Texas) 109.

Another potential route by which donor antigen could reach the thymus after a renal allograft might be by indirect presentation of that antigen on the surface of host antigen presenting cells which could pick up antigen in the transplant and then carry it to the thymus. In the case of the class II matched transplants studied here, this possibility is particularly attractive for the CD4 helper T cell pathway, since host and donor share the same class II antigens. Thus, induction of tolerance to host class II plus allogeneic peptides might produce tolerance to the same allogeneic determinants which would be encountered on donor antigen presenting cells in the graft. This hypothesis would also suggest that the tolerance induced would be at the level of the T helper cells and not the CTL precursors, which would thus have the added advantage of explaining why CTLp are not eliminated following tolerance induction in these animals. In nonthymectomized animals such CTLp would remain unactivated due to tolerance at the helper T cell level, while in thymectomized animals helper cells would no longer be tolerized in the thymus and could provide sufficient cytokines to activate CTLp and induce rejection.

The data described herein also suggest, however, an active role in tolerance induction for cells leaving the thymus and returning to the allograft. Otherwise, since cells already in the graft would presumably not be affected by what happens locally in the thymus, it would be difficult to explain the paradoxical result of thymic removal leading to rejection. One might invoke immunoregulatory cell populations (erstwhile "suppressor cells") leaving the thymus to return to the transplant where they can inhibit rejection, and the thymus has indeed been cited as a source of such cells (Ascherson, G.M. et al. (1976) *Eur. J. Immunol.* 6:699; Uhteg, L.C. et al. (1985) *J. Immunol.* 135L1800-1805; Noga, S.J. et al. (1988) Transplant. Proc. 20:1057-1067;Yoshima, N. et al. (1985) *Transplantation* 40:384-389; Prud'homme,

more consistent with current immunologic thinking, is that the ratio of cells in the transplant producing different cytokines could markedly influence the overall nature of the local immune response, and that this ratio may be what is influenced by events in the thymus. In particular, Th1 clones producing IL-2 and IFNγ would be immunostimulatory with respect to transplant immunity, whereas Th2 clones secreting the cytokines IL-4 and IL-10 would be immunosuppressive of the same reactions (Mosmann, T.R. et al. (1986) *J. Immunol.* 136:2348-2357; Gajewski, T.F. et al. (1990) *J. Immunol.* 144:4110-4120). Since Th2 cells can inhibit IFNγ and IL-2 production by Thl clones, the net result of a low Thl/Th2 ratio might be local suppression of transplant rejection. In the present study, comparative analysis of the cytokines expressed in kidney allografts of completely vs. partially thymectomized animals lend support to this hypothesis. Partially thymectomized animals, which later became tolerant, were found to express high levels of IL-10 and low or absent levels of IFNγ during the early postoperative course, while completely thymectomized recipients, which rejected their allografts, showed the reverse cytokine expression pattern.

These results also lend additional support to the concept that rejection and tolerance induction are two related but separable phenomena. While CyA treatment is capable of delaying the rejection process, tolerance is not induced unless there is an intact thymic environment. When thymic function is compromised by removal of all but 20% to 30% of the tissue, induction of tolerance eventually occurs, but only after a severe, self-limited rejection crisis, again suggesting an active process involving the thymus in the early phase of tolerance induction. The role of the thymus in maintaining tolerance long-term is less clear, since even complete thymectomy late in the course does not break the tolerance.

All of the pigs utilized in these studies have been sufficiently young to be expected to still have large, cellular thymuses. However, since pediatric cases constitute only a small fraction of current clinical transplantation, the majority of patients to whom these results could be applicable would be expected to have already passed the stage of thymic involution.

The activity (or number or concentration) of the cells and/or thymic products responsible for the promotion of tolerance can be maintained, or enhanced, to promote tolerance. This may be particularly important in older subjects.

The induction of tolerance with bone marrow transplantation

The following procedure was designed to lengthen the time an implanted organ (a xenograft) survives in a xenogeneic host prior to rejection. The organ can be any organ, e.g., a liver, e.g., a kidney, e.g., a heart. The main strategies are elimination of natural antibodies by organ perfusion, transplantation of tolerance-inducing bone marrow, optionally, the implantation of donor stromal tissue, and the administering growth hormone, to enhance host or donor thymus function (and optionally, in the case of a male recipient administration of a drug which mimics orchiemectomy).

Preparation of the recipient for transplantation includes any or all of these steps.

First, a preparation of horse anti-human thymocyte globulin (ATG) is intravenously injected into the recipient. The antibody preparation eliminates mature T cells and natural killer cells. If not eliminated, mature T cells would promote rejection of both the bone marrow transplant and, after sensitization, the xenograft itself. Of equal importance, the ATG preparation also eliminates natural killer (NK) cells. NK cells probably have no effect on the implanted organ, but would act immediately to reject the newly introduced bone marrow. Anti-human ATG obtained from any mammalian host can also be used, e.g., ATG produced in pigs, although thus far preparations of pig ATG have been of lower titer than horse-derived ATG. ATG is superior to anti-NK monoclonal Antibodies, as the latter are generally not lytic to all host NK cells, while the polyclonal mixture in ATG is capable of lysing all host NK cells. Anti-NK monoclonal antibodies can, however, be used.

The presence of donor antigen in the host thymus during the time when host T cells are regenerating post-transplant is critical for tolerizing host T cells. If donor hematopoietic stem cells are not able to become established in the host thymus and induce tolerance before host T cells regenerate repeated doses of anti-recipient T cell antibodies may be necessary throughout the non-myeloablative regimen. Continuous depletion of host T cells may be required for several weeks. Alternatively, e.g. if this approach is not successful, and tolerance (as measured by donor skin graft acceptance, specific cellular hyporesponsiveness *in vitro*, and humoral tolerance) is not induced in these animals, the approach can be modified to include host thymectomy and implantation of donor thymus.. In thymectomized recipients, host T cells do not have an opportunity to differentiate in a host thymus, but must differentiate in the donor thymus. If this is not possible, then the animal has to rely on donor T cells developing in the donor thymus for immunocompetence. Immunocompetence can be measured by the ability to reject a non-donor type allogeneic donor skin graft, and to survive in a pathogen-containing environment.

It may also be necessary or desirable to splenectomize the recipient in order to avoid anemia.

Second, the recipient is administered low dose radiation in order to create hematopoietic space for newly injected bone marrow cells. A sublethal dose of between 100 rads and 400 rads whole body radiation, plus 700 rads of local thymic radiation, has been found effective for this purpose.

Third, natural antibodies are absorbed from the recipient's blood by hemoperfusion of a liver of the donor species. Pre-formed natural antibodies (nAB) are the primary agents of graft rejection. Natural antibodies bind to xenogeneic endothelial cells and are primarily of the IgM class. These antibodies are independent of any known previous exposure to antigens of the xenogeneic donor. B cells that produce these natural antibodies tend to be T cell-independent, and are normally tolerized to self antigen by exposure to these antigens during development. The mechanism by which newly developing B cells are tolerized is unknown.

The fourth step in the non-myeloablative procedure is to implant donor stromal tissue, preferably obtained from fetal liver, thymus, and/or fetal spleen, into the recipient, preferably in the kidney capsule. Stem cell engraftment and hematopoiesis across disparate species barriers is enhanced by providing a hematopoietic stromal environment from the donor species. The stromal matrix supplies species-specific factors that are required for interactions between hematopoietic cells and their stromal environment, such as hematopoietic growth factors, adhesion molecules, and their ligands.

As liver is the major site of hematopoiesis in the fetus, fetal liver can also serve as an alternative to bone marrow as a source of hematopoietic stem cells. The thymus is the major site of T cell maturation. Each organ includes an organ specific stromal matrix that can support differentiation of the respective undifferentiated stem cells implanted into the host. Although adult thymus may be used, fetal tissue obtained sufficiently early in gestation is preferred because it is free from mature T lymphocytes which can cause GVHD. Fetal tissues also tend to survive better than adult tissues when transplanted. As an added precaution against GVHD, thymic stromal tissue can be irradiated prior to transplantation, e.g., irradiated at 1000 rads. As an alternative or an adjunct to implantation, fetal liver cells can be administered in fluid suspension.

Fifth, bone marrow cells (BMC), or another source of hematopoietic stem cells, e.g., a fetal liver suspension, of the donor are injected into the recipient. Donor BMC home to appropriate sites of the recipient and grow contiguously with remaining host cells and proliferate, forming a chimeric lymphohematopoietic population. By this process, newly forming B cells (and the antibodies they produce) are exposed to donor antigens, so that the transplant will be recognized as self. Tolerance to the donor is also observed at the T cell level in animals in which hematopoietic stem cell, e.g., BMC, engraftment has been achieved. When an organ graft is placed in such a recipient several months after bone marrow chimerism has been induced, natural antibody against the donor will have disappeared, and the graft should be accepted by both the humoral and the cellular arms of the immune system. This approach has the added advantage of permitting organ transplantation to be performed sufficiently long following transplant of hematopoietic cells, e.g., BMT, e.g., a fetal liver suspension, that normal health and immunocompetence will have been restored at the time of organ transplantation. The use of xenogeneic donors allows the possibility of using bone marrow cells and organs from the same animal, or from genetically matched animals.

Recipient (or donor) thymic activity can be enhanced by the administration of growth hormone or (if the recipient is a male), administering a substance, e.g., a drug which mimics orchiemectomy. Administration can begin prior to, at the time of, or after, implantation of donor tissue. A useful dosage and course of administration of an administered substance can be determined by trials in a model system (e.g., one of the systems for the induction of tolerance described in one of the above referenced U.S. patent applications) in which the

While any of these procedures may aid the survival of an implanted organ, best results are achieved when all steps are used in combination. Methods of the invention can be used to confer tolerance to allogeneic grafts, e.g., wherein both the graft donor and the recipient are humans, and to xenogeneic grafts, e.g., wherein the graft donor is a nonhuman animal, e.g., a swine, e.g., a miniature swine, and the graft recipient is a primate, e.g., a human.

In the case of xenogeneic grafts, the donor of the implant and the individual that supplies either the tolerance-inducing hematopoietic cells or the liver to be perfused should be the same individual or should be as closely related as possible. For example, it is preferable to derive implant tissue from a colony of donors that is highly inbred.

The methods of the invention may be employed with other mammalian recipients (e.g., rhesus monkeys) and may use other mammalian donors (e.g., primates, sheep, or dogs). As an alternative or adjunct to hemoperfusion, host antibodies can be depleted by administration of an excess of hematopoietic cells.

Stromal tissue introduced prior to hematopoietic cell transplant, e.g., BMT, may be varied by: (1) administering the fetal liver and thymus tissue as a fluid cell suspension; (2) administering fetal liver or thymus stromal tissue but not both; (3) placing a stromal implant into other encapsulated, well-vascularized sites, or (4) using adult thymus or fetal spleen as a source of stromal tissue.

OTHER EMBODIMENTS

The methods described herein for inducing tolerance to, or promoting the acceptance of, an allogeneic antigen or allogeneic graft can be used where, as between the donor and recipient, there is any degree of mismatch at MHC loci or other loci which influence graft rejection. Preferably, there is a mismatch at least one MHC locus or at least one other locus that mediates recognition and rejection, e.g., a minor antigen locus. With respect to class I and class II MHC loci, the donor and recipient can be: matched at class I and mismatched at class II; mismatched at class I and matched at class II; mismatched at class I and mismatched at class II; matched at class I, matched at class II. In any of these combinations other loci which control recognition and rejection, e.g., minor antigen loci, can be matched or mismatched. As stated above, it is preferable that there is mismatch at least one locus. Mismatched at MHC class I means mismatched for one or more MHC class I loci, e.g., in the case of humans, mismatched at one or more of HLA-A, HLA-B, or HLA-C, or in the case of swine, mismatch at one or more SLA class I loci, e.g., the swine A or B loci. Mismatched at MHC class II means mismatched at one or more MHC class II loci, e.g., in the case of humans, mismatched at one or more of a DP α, a DPβ, a DQ α, a DQ β, a DR α, or a DR β, or in the case of swine, mismatch at one or SLA class II loci, e.g., mismatch at DQ α or β, or DR α or β.

The methods described herein for inducing tolerance to an allogeneic antigen or allogeneic graft can be used where, as between the donor and recipient, there is any degree of

mixed lymphocyte reactivity between the donor and the recipient. In preferred embodiments mixed lymphocyte reactivity is used to define mismatch for class II, and the invention includes methods for performing allogeneic grafts between individuals with any degree of mismatch at class II as defined by a mixed lymphocyte assay. Serological tests can be used to determine mismatch at class I or II loci and the invention includes methods for performing allogeneic grafts between individuals with any degree of mismatch at class I and or II as measured with serological methods. In a preferred embodiment, the invention features methods for performing allogeneic grafts between individuals which, as determined by serological and or mixed lymphocyte reactivity assay, are mismatched at both class I and class II.

The methods of the invention are particularly useful for replacing a tissue or organ afflicted with a neoplastic disorder, particularly a disorder which is resistant to normal modes of therapy, e.g., chemotherapy or radiation therapy. Methods of the invention can be used for inducing tolerance to a graft, e.g., an allograft, e.g., an allograft from a donor which is mismatched at one or more class I loci, at one or more class II loci, or at one or more loci at each of class I and class II. In preferred embodiments: the graft includes tissue from the digestive tract or gut, e.g., tissue from the stomach, or bowel tissue, e.g., small intestine, large intestine, or colon; the graft replaces a portion of the recipient's digestive system e.g., all or part of any of the digestive tract or gut, e.g., the stomach, bowel, e.g., small intestine, large intestine, or colon.

Tolerance, as used herein, refers not only to complete immunologic tolerance to an antigen, but to partial immunologic tolerance, i.e., a degree of tolerance to an antigen which is greater than what would be seen if a method of the invention were not employed.

Some of the methods described herein use lethal irradiation to create hematopoietic space, and thereby prepare a recipient for the administration of xenogeneic, stem cells. In any of the methods described herein, particularly primate or clinical methods, it is preferable to create hematopoietic space for the administration of such cells by non-lethal means, e.g., by administering sub-lethal doses of irradiation, bone marrow depleting drugs, or antibodies. The use of sublethal levels of bone marrow depletion allows the generation of mixed chimerism in the recipient. Mixed chimerism is generally preferable to total or lethal ablation of the recipient bone marrow followed by complete reconstitution of the recipient with administered stem cells.

As is discussed herein, it is often desirable to expose a graft recipient to irradiation in order to promote the development of mixed chimerism. It is possible to induce mixed chimerism with less radiation toxicity by fractionating the radiation dose, i.e., by delivering the radiation in two or more exposures or sessions. Accordingly, in any method of the invention calling for the irradiation of a recipient, e.g., a primate, e.g., a human, recipient, of a xenograft or allograft, the radiation can either be delivered in a single exposure, or more

fractionated dosages is preferably equal, e.g., in rads or Gy, to the radiation dosage which can result in mixed chimerism when given in a single exposure. The fractions are preferably approximately equal in dosage. For example, a single dose of 700 rads can be replaced with, e.g., two fractions of 350 rads, or seven fractions of 100 rads. Hyperfractionation of the radiation dose can also be used in methods of the invention. The fractions can be delivered on the same day, or can be separated by intervals of one, two, three, four, five, or more days. Whole body irradiation, thymic irradiation, or both, can be fractionated.

Much or all of the preparative regimen can be delivered or administered to a recipient, e.g., an allograft or xenograft recipient, within a few days, preferably within 72, 48, or 24 hours, of transplantation of tolerizing stem cells and/or the graft. This is particularly useful in the case of humans receiving grafts from cadavers. Accordingly, in any of the methods of the invention calling for the administration of treatments prior to the transplant of stem cells and/or a graft, e.g., treatments to inactivate or deplete host antibodies, treatments to inactivate host T cells or NK cells, or irradiation, the treatment(s) can be administered, within a few days, preferably within 72, 48, or 24 hours, of transplantation of the stem cells and/or the graft. In particular, primate, e.g., human, recipients of allografts can be given any or all of treatments to inactivate or deplete host antibodies, treatments to inactivate host T cells or NK cells, or irradiation, within a few days, preferably within 72, 48, or 24 hours, of transplantation of stem cells and/or the graft. For example, treatment to deplete recipient T cells and/or NK cells, e.g., administration of ATG, can be given on day -2, -1, and 0, and WBI, thymic irradiation, and stem cell, e.g., bone marrow stem cells, administered on day 0. (The graft, e.g., a renal allograft, is transplanted on day 0).

Methods of the invention can include recipient splenectomy.

As is discussed herein, hemoperfusion, e.g., hemoperfusion with a donor organ, can be used to deplete the host of natural antibodies. Other methods for depleting or otherwise inactivating natural antibodies can be used with any of the methods described herein. For example, drugs which deplete or inactivate natural antibodies, e.g., deoxyspergualin (DSG) (Bristol), or anti-IgM antibodies, can be administered to the recipient of an allograft or a xenograft. One or more of, DSG (or similar drugs), anti-IgM antibodies, and hemoperfusion, can be used to deplete or otherwise inactivate recipient natural antibodies in methods of the invention. DSG at a concentration of 6 mg/kg/day, i.v., has been found useful in suppressing natural antibody function in pig to cynomolgus kidney transplants.

As is discussed in PCT/US94/01616, hereby incorporate by reference, the engraftment of exogenously supplied hematopoietic stem cells can be promoted by treating the recipient of the cells so as to induce hematopoietic space in the recipient. Hematopoietic space is commonly induced by radiation, but other procedures can replace or reduce the need for WBI. For example, space can be created by treating the recipient with a monoclonal antibody against MHC class I antigens expressed by the recipient (see e.g., Voralia, M. et al. (1987)

myelosuppressive drugs (see e.g., Lapidot, T. et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:4595). As for WBI, space created within the recipient for bone marrow transplantation by other mechanisms (e.g., anti-MHC class I treatment or myelosuppressive drugs) can be assessed by monitoring WBC counts in the recipient.

Alternative methods for the inactivation of thymic T cells are also included in embodiments of the invention. Some of the methods described herein include the administration of thymic irradiation to inactivate host thymic-T cells or to otherwise diminish the host's thymic-T cell mediated responses to donor antigens. It has been discovered that the thymic irradiation called for in allogeneic or xenogeneic methods of the invention can be supplemented with, or replaced by, other treatments which diminish (e.g., by depleting thymic-T cells and/or down modulating one or more of the T cell receptor (TCR), CD4 co-receptor, or CD8 co-receptor) the host's thymic-T cell mediated response. For example, thymic irradiation can be supplemented with, or replaced by, anti-T cell antibodies (e.g., anti-CD4 and/or anti-CD8 monoclonal antibodies) administered a sufficient number of times, in sufficient dosage, for a sufficient period of time, to diminish the host's thymic-T cell mediated response.

For best results, anti-T cell antibodies should be administered repeatedly. E.g., anti-T cell antibodies can be administered one, two, three, or more times prior to donor bone marrow transplantation. Typically, a pre-bone marrow transplantation dose of antibodies will be given to the patient about 5 days prior to bone marrow transplantation. Additional, earlier doses 6, 7, or 8 days prior to bone marrow transplantation can also be given. It may be desirable to administer a first treatment then to repeat pre-bone marrow administrations every 1-5 days until the patient shows excess antibodies in the serum and about 99% depletion of peripheral T cells and then to perform the bone marrow transplantation. Anti-T cell antibodies can also be administered one, two, three, or more times after donor bone marrow transplantation. Typically, a post-bone marrow transplant treatment will be given about 2-14 days after bone marrow transplantation. The post bone marrow administration can be repeated as many times as needed. If more than one administration is given the administrations can be spaced about 1 week apart. Additional doses can be given if the patient appears to undergo early or unwanted T cell recovery. Preferably, anti-T cell antibodies are administered at least once (and preferably two, three, or more times) prior to donor bone marrow transplantation and at least once (and preferably two, three, or more times) after donor bone marrow transplantation.

As described in PCT/US94/01616, hereby incorporated by reference, it has been discovered that there is a permissible time period ("window") for hematopoietic stem cell engraftment following the creation of space (e.g., by whole body irradiation) for the donor hematopoietic stem cells in a recipient. It has further been discovered that space created for hematopoietic stem cell engraftment can be monitored over time by monitoring peripheral

hematopoietic space generally results in a reduction in white blood cell (WBC) levels (as revealed, e.g., by WBC counts) and the WBC reduction serves as a marker for the presence of hematopoietic space. The marker is a conservative one since WBC counts may recover at a time when space is still present in an animal.

Accordingly, in any method which involves hematopoietic stem cell transplantation, and thus also requires the creation of hematopoietic space in a recipient, transplantation can be performed during the permissible window for engraftment following creation of space for the hematopoietic stem cells. Likewise, in any method in which space is created for exogenously administered hematopoietic stem cells, white blood cell levels can be followed to monitor space for the donor hematopoietic stem cells (i.e., to assess the permissible window for engraftment). Examples of procedures involving hematopoietic stem cell transplantation include: 1) conditioning of a recipient for an allo- or xenograft in which hematopoietic stem cell transplantation is performed in conjunction with transplantation of another allo- or xenograft; 2) treatment of various hematopoietic disorders, including leukemias, lymphomas and other hematopoietic malignancies and genetic hematopoietic disorders (e.g., adenosine deaminase deficiency, bare lymphocyte syndrome and other congenital immunodeficiency diseases) in which hematopoietic stem cell transplantation is performed therapeutically; and 3) transplantation of genetically modified hematopoietic stem cells (e.g., genetically modified autologous hematopoietic stem cells) to deliver a gene product to a recipient (e.g., as gene therapy).

Accordingly, methods of the invention include a method of determining if a myelosuppressive or hematopoietic-space inducing treatment is sufficient to create hematopoietic space. The method includes administering a myelosuppressive treatment to a recipient, and determining the level of white blood cells in the recipient, e.g., by determining the WBC count of the recipient, a depression in the level of white blood cells being indicative of the presence or induction of hematopoietic space.

Other embodiments are within the following claims.

What is claimed is:

1. A method of promoting, in a recipient mammal, acceptance of an allograft from a donor mammal, comprising:

inducing tolerance to said allograft;

implanting said allograft into said recipient; and

enhancing thymus function in said recipient.

2. The method of claim 1, wherein thymus function is enhanced by administering growth hormone to said recipient.

3. The method of claim 1, wherein the recipient is a male and thymus function is enhanced by administering a substance which mimics orchiemectomy to said recipient.

4. The method of claim 1, wherein said tolerance-inducing step comprises: administering to said recipient a short course of help reducing treatment.

5. The method of claim 1, wherein said tolerance-inducing step comprises: introducing hematopoietic stem cells of said donor into said recipient.

6. The method of claim 1, wherein said tolerance-inducing step comprises:

inserting DNA encoding an MHC antigen of said donor into a hematopoietic stem cell of said recipient; and

allowing the MHC antigen encoding DNA to be expressed in said recipient.

7. The method of claim 1, further comprising:

selecting said donor such that it is matched or tolerant of a mismatch at a first locus which affects graft rejection.

8. A method of promoting, in a recipient mammal, acceptance of a xenograft from a donor mammal, comprising:

inducing tolerance to said xenoograft;

implanting said xenograft into said recipient; and

enhancing thymus function in said recipient.

9. The method of claim 8, wherein thymus function is enhanced by administering growth hormone to said recipient.

10. The method of claim 8, wherein the recipient is a male and thymus function is

11. The method of claim 8, wherein said tolerance-inducing step comprises: introducing hematopoietic stem cells of said donor into said recipient.

12. The method of claim 8, wherein said tolerance-inducing step comprises:

inserting DNA encoding an MHC antigen of said donor into a hematopoietic stem cell of said recipient; and

allowing the MHC antigen encoding DNA to be expressed in said recipient.

13. A method of promoting, in a recipient mammal, acceptance of a heart allograft from a donor mammal, comprising:

inducing tolerance to said heart allograft;

implanting into said recipient said heart allograft ; and

maintaining thymus function in said recipient.

14. The method of claim 13, further comprising enhancing thymus function in said recipient.

15. The method of claim 14, further comprising enhancing thymus function by administering growth hormone to said recipient.

16. The method of claim 14, wherein said tolerance-inducing step comprises: administering to said recipient a short course of help reducing treatment.

17. The method of claim 13, wherein said tolerance-inducing step comprises: introducing hematopoietic stem cells of said donor into said recipient.

18. The method of claim 13, wherein the recipient is a male and thymus function is enhanced by administering a substance which mimics orchiemectomy to said recipient.

19. The method of claim 13, wherein said tolerance-inducing step comprises:

inserting DNA encoding an MHC antigen of said donor into a hematopoietic stem cell of said recipient; and

allowing the MHC antigen encoding DNA to be expressed in said recipient.

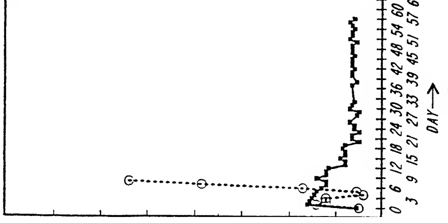


FIG. 1A

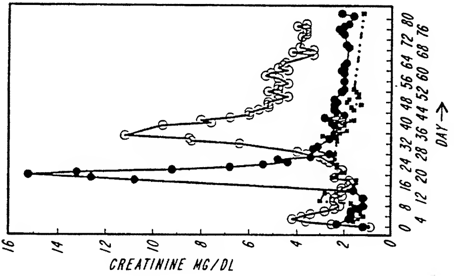


FIG. 1B

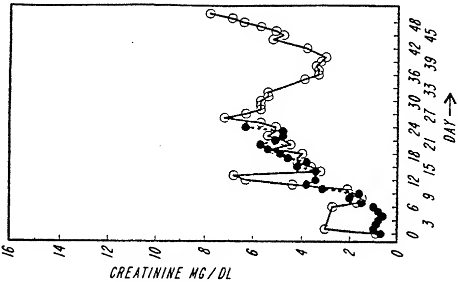
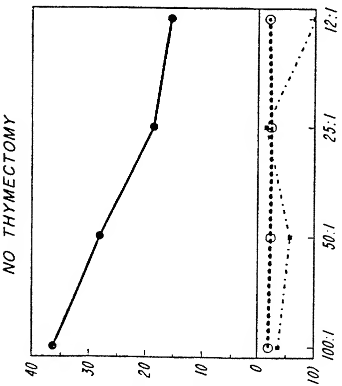


FIG. 1C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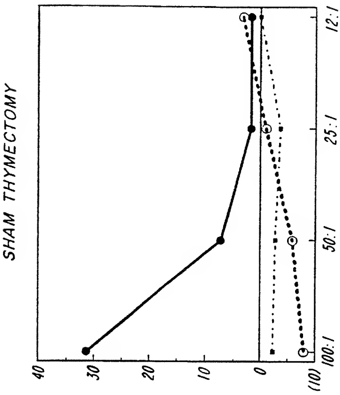
30
20
10
0
(10)
100:1
50:1
25:1
12:1

FIG. 2A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(10)
100:1
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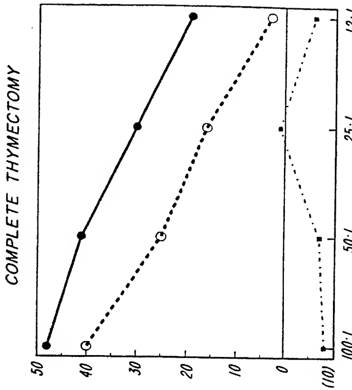
12:1

FIG. 2B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30
20
10
0
(10)
100:1
50:1
25:1
12:1

FIG. 2D

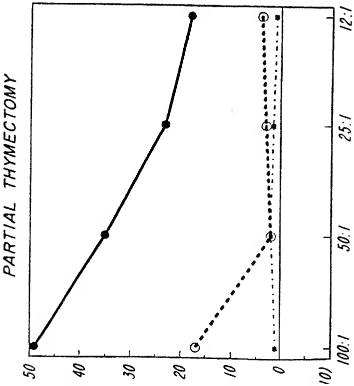
PARTIAL THYMECTOMY
50
40
30
20
10
0
(10)
100:1
50:1
25:1
12:1

FIG. 2C

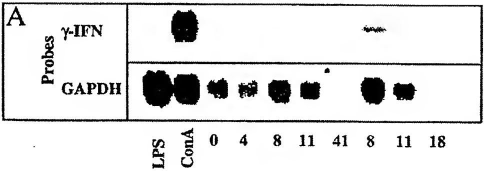

FIG. 3A

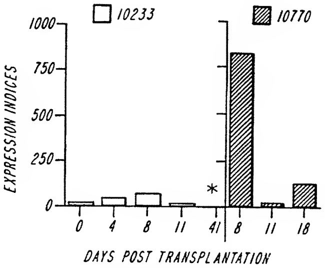
10233
10770
1000
750
500
250
0
EXPRESSION INDICES
*
0
4
8
11
41
8
11
18
DAYS POST TRANSPLANTATION

FIG. 3B

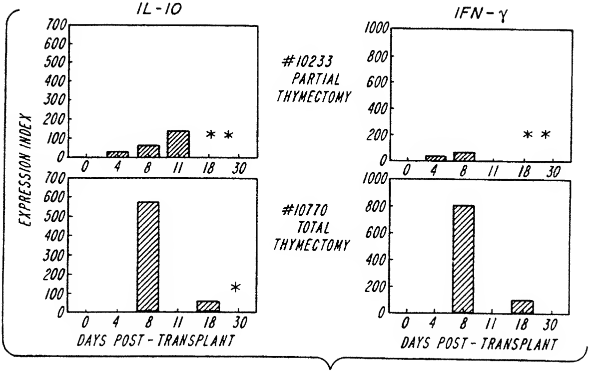
IL-10
IFN-γ
EXPRESSION INDEX
#10233
PARTIAL
THYMECTOMY
#10770
TOTAL
THYMECTOMY
700
600
500
400
300
200
100
0
1000
800
600
400
200
0
4
8
11
18
30
* *
*
DAYS POST-TRANSPLANT
DAYS POST-TRANSPLANT

FIG. 4A

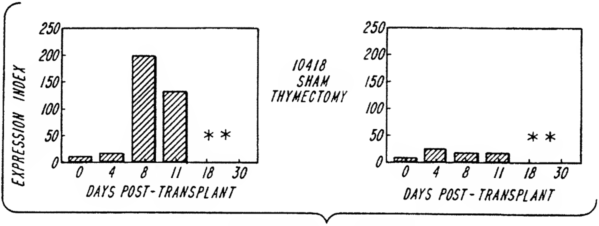
EXPRESSION INDEX
250
200
150
100
50
0
* *
0 4 8 11 18 30
DAYS POST-TRANSPLANT
10418
SHAM
THYMECTOMY
250
200
150
100
50
0
* *
0 4 8 11 18 30
DAYS POST-TRANSPLANT

FIG. 4B

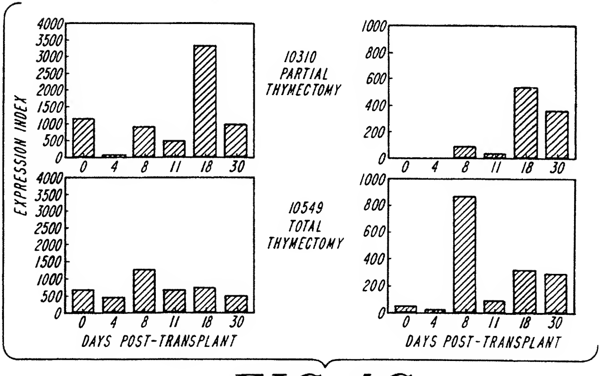
EXPRESSION INDEX
4000
3500
3000
2500
2000
1500
1000
500
0
0 4 8 11 18 30
10310
PARTIAL
THYMECTOMY
1000
800
600
400
200
0
0 4 8 11 18 30
4000
3500
3000
2500
2000
1500
1000
500
0
0 4 8 11 18 30
DAYS POST-TRANSPLANT
10549
TOTAL
THYMECTOMY
1000
800
600
400
200
0
0 4 8 11 18 30
DAYS POST-TRANSPLANT

FIG. 4C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/10598

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 48/00
US CL :424/93.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, Dialog
search terms: tolerance, transplantation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Proceedings of the National Academy of Sciences, Volume 87, issued June 1990, Lapidot et al., "Enhancement of bone marrow allografts from nude mice into mismatched recipients by T cells void of graft-versus host activity", pages 4595-4599, see entire reference.	1, 5 , 7, 15, 16 and 19
Y	THE LANCET, Volume 123, issued 03 June 1978, Green et al., "Extensive prolongation of rabbit kidney allgraoft survival after short-term cyclosporin-A treatment", pages 1182-1183, see entire reference.	2-4 and 8-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be part of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 OCTOBER 1995	20 NOV 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Suzanne Ziska, Ph.D.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Transplantation Proceedings, Volume 23, No. 1, issued February 1991, Fischel et al., "Prolonged Survival of a Discordant Cardiac Xenograft in a Rhesus Monkey", pages 589-590, see entire reference.	2-4 and 8-10
Y	European Journal of Immunology, Volume 6, issued 1976, Asherson et al., "Adult thymectomy prevention of the appearance of suppressor T cells which depress contact sensitivity to picryl chloride and reversal of adult thymectomy effect by thymus extract", pages 699-703, see entire reference.	2-4 and 8-10
Y	Trends in Genetics, issued June 1986, Dick et al., "Genetic manipulation of hematopoietic stem cells with retrovirus vectors", pages 165-170, see entire reference.	6, 11, 12, 18 and 20
Y	Transplantation Proceedings, Volume 21, No, 1, issued February 1989, Madsen et al., "Induction of Immunological Unresponsiveness Using Recipient Cells Transfected with Donor Class I or Class II MHC Genes", page 477, see entire reference.	6,11, 12, 18 and 20
X	The Journal of Immunology, Volume 135, No. 3, issued 03 September 1985, Uhteg et al., "Cyclosporine-induced transplantation unresponsiveness is rat cardiac allograft recipients: in vitro determination of helper and suppressor activity", pages 1800-1805, see entire reference.	14 and 17